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A novel flavonoid isolated from the steam-bark of *Ulmus Wallichiana* Planchon stimulates osteoblast function and inhibits osteoclast and adipocyte differentiation ☆

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ABSTRACT

(2S,3S)-Aromadendrin-6-C-β-D-glucopyranoside (AG) is a novel flavonol isolated from the extract of *Ulmus wallichiana* (Himalayan Elm). Extract of *U. wallichiana* is used as a traditional medicine for rapid fracture repair in India. We characterized the mechanism of action of AG in mouse bone cells by investigating its effect on the precursors of osteoblasts, osteoclasts and adipocytes. At nanomolar concentrations, AG increased differentiation of preosteoblasts obtained from neonatal mouse calvaria. The gene expression of osteogenic markers, including runt-related transcription factor 2 (Runx-2), bone morphogenetic protein-2 (BMP-2), type I collagen and osteocalcin were elevated in the preosteoblasts. The extracellular matrix mineralization was higher in preosteoblast and bone marrow cells when AG was present in the medium. Furthermore, AG protected the differentiated osteoblasts from serum deprivation-induced apoptosis, and increased the expression of the anti-osteoclastogenic cytokine, osteoprotegerin. It inhibited osteoclast differentiation of bone marrow precursor cells to osteoclasts in the presence of receptor activator of nuclear factor kappa-B ligand (RANKL) and monocyte/macrophage-colony stimulating factor (M-CSF). Additionally, in 3T3-L1 preadipocytes, AG decreased the expression of genes involved in adipogenesis, including peroxisome proliferator-activated receptor gamma (PPARγ), sterol regulatory element binding protein (SREBP) and CCAAT/enhancer-binding protein alpha (CEBP/α), and induced apoptosis of differentiated adipocytes. Induction of adipogenic differentiation was also inhibited in the presence of AG. AG exhibited no estrogenic/antiestrogenic effect. Together, our data show that AG has potent osteogenic, anti-osteoclastogenic and anti-adipogenic effects, which may translate to a better skeletal outcome in postmenopausal osteoporosis.

1. Introduction

Postmenopausal osteoporosis is a disease in which there is net bone loss because of an imbalance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation (Raisz and Rodan, 2003). One of the strategies for treating it is through inhibition of bone resorption by osteoclasts and/or increase in bone formation by osteoblasts. After menopause mesenchymal stem cells (MSCs) in the bone marrow are differentiated more to adipocytes and less to osteoblasts, affecting bone formation as shown by various clinical studies and other reports (Burkhardt et al., 1987; Meunier et al., 1971; Sekiya et al., 2004; Verma et al., 2002). Increased adipocytes in the bone marrow result in increased production of adipokines including monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6) that stimulate osteoclast differentiation and functions (Christiansen et al., 2005; Cirmanova et al., 2008; Ebina et al., 2008; Laharrague et al., 2000; Soltan et al., 2009). Therefore, inhibition of adipogenesis in the bone marrow with a concomitant increase in osteoblastogenesis and decrease in osteoclastogenesis is considered to be a therapeutic strategy for osteoporosis.

A growing body of literature suggests bone conserving effects of plant-derived polyphenolic compounds in the settings of estrogen deficiency-induced and aging-induced bone loss. Studies showed that different polyphenols can stimulate osteoblast function, and inhibit osteoclast and adipocyte functions either alone or in combination. Due to their natural occurrence and lack of side effects, phytochemicals are considered to be safer than the conventional hormone/estrogen replacement therapy as preventive measures against various diseases including osteoporosis. However, high concentrations (micromolar) of flavonoids have mostly been used in studies with bone cells *in vitro* and these concentrations are not likely to be achieved *in vivo*, particularly at the bone tissue level (Sharan et al., 2009). Lately, efforts to identify

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more potent analogs of flavonoids with respect to their effects on bone cells have intensified (Setchell and Lydeking-Olsen, 2003; Sharan et al., 2009).

In our search for more potent flavonoid analog(s), we embarked on the isolation of bioactive compounds from a butanolic fraction from the stem-bark of *U. wallichiana* (Himalayan Elm). Stem-bark extract of *U. wallichiana* is known in Indian traditional medicine to accelerate fracture repair (Sharan et al., 2010a). The beneficial outcomes of stem-bark extracts of *U. wallichiana* on bone appear to be due to the presence of several flavonoid-*C*-glucoside compounds (Sharan et al., 2010a,b; Siddiqui et al., 2011a). Flavonoid-*C*-glucosides have much scarce occurrence in nature compared to aglycone and *O*-glucoside flavonoids, and very little is known about their pharmacological effects. In flavonoid-*C*-glucosides, the sugar is directly linked to the flavonoid nucleus via an acid-resistant and largely enzyme-resistant C—C bond, thereby augmenting metabolic stability and oral bioavailability over their aglycone or *O*-glucoside counterparts (Prasain and Barnes, 2007; Siddiqui et al., 2011a). During isolation and characterization of abundantly present compounds from stem-bark extracts of *U. wallichiana*, we isolated (2S,3S)-aromadendrin-6-*C*-β-D-glucopyranoside (AG) in a pure form (Rawat et al., 2009). Chemical characterization revealed that AG is a novel compound belonging to flavonol class.

We hypothesized that AG could be a bioactive compound contributing to the osteoprotective effect of butanolic fraction made from the stem-bark extracts of *U. wallichiana* (Rawat et al., 2009; Sharan et al., 2010a). The present study is designed to characterize the effects of AG on osteoblastogenesis, osteoclastogenesis and adipogenesis using different established murine culture models that had been differentiated into osteoblasts, osteoclasts and adipocytes.

2. Materials and methods

2.1. Reagents and chemicals

Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA). All fine chemicals were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Culture of calvarial osteoblasts

Mice calvarial osteoblasts were obtained from previously published protocol of sequential digestion (Trivedi et al., 2009; Wong et al., 1978). Briefly, calvaria from ten to twelve 1- to 2-day-old *Balb/c* mice were pooled. Following surgical isolation from the skull and the removal of sutures and adherent mesenchymal tissues, the calvaria was subjected to five sequential (10 to 15 min) digestions at 37 °C in a solution containing 0.1% dispase and 0.1% collagenase P. The cells from the second to fifth digestions were collected, centrifuged, resuspended, and plated in a T-25 cm^2 flask in α-MEM containing 10% FBS and 1% penicillin/streptomycin.

2.3. Osteoblast differentiation and mineralization

For determination of alkaline phosphatase (ALP) activity, 2×10^3 cells/well were seeded in 96-well plates. Cells were treated with different concentrations of the AG for 48 h in α-MEM supplemented with 5% charcoal treated FBS, 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid and 1% penicillin/streptomycin (osteoblast differentiation medium). At the end of the incubation period, total ALP activity was measured using p-nitrophenylphosphate (PNPP) as a substrate and quantitated colorimetrically at 405 nm (Bhargavan et al., 2009; Gautam et al., 2010; Trivedi et al., 2009; Trivedi et al., 2008). For mineralization studies 10×10^3 cells/well were seeded in 12-well plates in differentiation media with 10% charcoal treated FBS. Cells were cultured with and without AG for 21 days at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, and the medium was changed every 48 h. After 21 days, the attached cells were fixed in 4% formaldehyde for 20 min at room temperature and rinsed once in PBS. After fixation, the specimens were processed for staining with 40 mM Alizarin Red-S, which stains areas rich in nascent calcium.

For quantification of Alizarin Red-S staining, 800 μl of 10% (v/v) acetic acid was added to each well, and plates were incubated at room temperature for 30 min with shaking. The monolayer, now loosely attached to the plate, was then scraped with a cell scraper and transferred with 10% (v/v) acetic acid to a 1.5-ml tube. After vortexing for 30 s, the slurry was overlaid with 500 μl mineral oil (Sigma-Aldrich), heated to exactly 85 °C for 10 min, and transferred to ice for 5 min. The slurry was then centrifuged at 20,000×*g* for 15 min and 500 μl of the supernatant was removed to a new tube. Then 200 μl of 10% (v/v) ammonium hydroxide was added to neutralize the acid. In some cases, the pH was measured at this point to ensure that it was between 4.1 and 4.5. The OD (405 nm) of 150 μl aliquots of the supernatant was measured in 96-well format using opaque-walled, transparent-bottomed plates (Bhargavan et al., 2009; Gautam et al., 2010; Gregory et al., 2004; Maurya et al., 2009).

2.4. Differentiation and mineralization of bone marrow cells

Bone marrow cells from 4–6 weeks old female *Balb-c* mice were isolated and cultures were prepared according to a previously published protocol (Maniatopoulos et al., 1988). Briefly, the femora were excised aseptically, cleaned of soft tissues, and washed three times, 15 min each, in a culture medium. The epiphyses of femora were cut off and the marrow was flushed out in 20 ml of a culture medium consisting of α-MEM, supplemented with 10% fetal bovine serum (FBS), 10^{-7}M dexamethasone, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate. Released bone marrow cells were collected and plated (2×10^6 cells/well) in 12-well plate in the culture medium, consisting of α-MEM, supplemented with 10% fetal bovine serum, 10^{-7}M dexamethasone, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate. Cells were cultured with and without AG for 21 days at 37 °C in a humidified atmosphere of 5% CO2 and 95% air, and the medium was changed every 48 h. After 21 days, the attached cells were fixed in 4% formaldehyde for 20 min at room temperature and rinsed once in PBS. After fixation, the specimens were processed for staining with 40 mM Alizarin Red-S, which stains areas rich in nascent calcium.

For differentiation the cells were cultured for nine days in differentiation conditions. At the end of incubation period, total ALP activity was measured using p-nitrophenylphosphate (PNPP) as substrate and quantitated colorimetrically at 405 nm.

2.5. Induction of adipogenic differentiation in 3T3-L1 cells

Two-day post confluency 3T3-L1 cells were treated with the induction media [10% FBS in DMEM containing 1 μg/ml insulin, 1 μM dexamethasone and 500 μM isobutylmethylxanthine]. Two days after induction medium treatment (day 2), the cells were treated with insulin alone (10% FBS in DMEM containing 1 μg/ml insulin). Full differentiation was usually achieved after eight days from day 0. Effect of different concentrations of AG on the differentiation of 3T3-L1 preadipocytes to adipocytes was observed. For assessment of adipogenesis the differentiated cells were fixed in 4% paraformaldehyde w/v for 20 min, washed with PBS and stained with 0.34% Oil Red O in 60% isopropanol for 15 min. Then it was washed with PBS thrice and stain was extracted with 80% isopropanol by keeping it at room temperature for 30 min on an orbital shaker. OD of the extracted dye was taken at 520 nm.

2.6. Induction of adipogenic differentiation in bone marrow cells

For adipogenic differentiation, 1×10^7 bone marrow cells were seeded in 24-well plates and cultured in an adipogenic medium consisting of